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A Single, Mild, Transient Scrotal Heat Stress Causes Hypoxia and Oxidative Stress in Mouse Testes, Which Induces Germ Cell Death[1]

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ABSTRACT

Spermatogenesis is a temperature-dependent process, and increases in scrotal temperature can disrupt its progression. We previously showed that heat stress causes DNA damage in germ cells, an increase in germ cell death (as seen on TUNEL staining), and subfertility. The present study evaluated the stress response in mouse testes following a single mild transient scrotal heat exposure (40°C or 42°C for 30 min). We investigated markers of three types of stress response, namely, hypoxia, oxidative stress, and apoptosis. Heat stress caused an increase in expression of hypoxia-inducible factor 1 alpha (*Hif1a*) mRNA expression and translocation of HIF1A protein to the germ cell nucleus, consistent with hypoxic stress. Increased expression of heme oxygenase 1 (*Hmox1*) and the antioxidant enzymes glutathione peroxidase 1 (GPX1) and glutathione S-transferase alpha (GSTA) was consistent with a robust oxidative stress response. Germ cell death was associated with an increase in expression of the effector caspase cleaved caspase 3 and a decrease in expression of the protein inhibitor of caspase-activated DNase (ICAD). Reduced expression of ICAD contributes to increased activity of caspase-activated DNase and is consistent with the increased rates of DNase fragmentation that have been detected previously using TUNEL staining. These studies confirmed that transient mild testicular hyperthermia results in temperature-dependent germ cell death and demonstrated that elevated temperature results in a complex stress response, including induction of genes associated with oxidative stress and hypoxia.

apoptosis, spermatogenesis, stress, testis

INTRODUCTION

The importance of thermoregulation in the testis is illustrated by the fact that slight increases in temperature can disrupt spermatogenesis and ultimately cause problems with fertility [1–6]. The testis displays a variety of mechanisms that are triggered on exposure to stress, including DNA repair, heat shock response, oxidative stress response, and apoptosis and cell death. Testes in most mammals are found in the scrotum outside the main body cavity and are thus 2–8°C below core body temperature [7, 8]. In addition, the temperature within the testis is regulated by a countercurrent heat exchange system between the pampiniform plexus and the testicular artery. Any disruption to this system may cause problems with spermatogenesis.

Previous findings have suggested that increased metabolism in the testis after heat stress may not be met by a sufficient increase in blood flow [9]. Thus, there is a possibility of the testis becoming hypoxic [10]. Hypoxia occurs when the oxygen tension drops below that required for normal cellular function in a particular tissue. This can be in response to inadequate blood flow into the tissue or reduced oxygen transport capacity [11]. Hypoxia has been shown to result in cell cycle arrest and apoptosis [12, 13]. Hypoxia-inducible factor (HIF) is composed of α and β (ARNT) subunits, which dimerize under hypoxic conditions [14]. Activation of HIF during hypoxic conditions leads to HIF1A binding to specific response elements in target genes involved in vasodilation, angiogenesis, and glycolysis [15, 16].

The production of free radicals and reactive oxygen species (ROS), including the superoxide anion and hydrogen peroxide, can induce positive changes in sperm function, including hyperactivation, capacitation, and the acrosome reaction [17]. However, high levels of these species can be deleterious and cause oxidative damage to DNA, and there have been several studies [18–20] linking oxidative stress to male fertility problems. The testis contains a number of antioxidant proteins that serve to protect germ cells from oxidative damage. These include superoxide dismutase (SOD), glutathione reductase (GSR), glutathione peroxidase (GPX), glutathione S-transferase (GST), and heme oxygenase 1 (HMOX1) [21, 22]. The heme oxygenase system has an important role in protecting cells from the deleterious effects of oxidative stress and consists of heme oxygenase proteins (inducible HMOX1 and constitutive HMOX2) [23].

In many cell types, hypoxia and oxidative stress have been shown to trigger apoptosis and cell death [24, 25]. One of the hallmarks of apoptosis is the fragmentation of DNA, thought to be mediated by caspase-activated DNase (CAD) and modulated by its inhibitor (ICAD), which inhibits CAD-induced degradation of nuclear DNA [26]. It is believed that caspase 3 cleaves ICAD to inactivate its inhibiting effect on CAD, thereby inducing DNA fragmentation in one of the final steps of apoptosis [27].

Several rodent models have been used to study the effect of heat stress on the testis, including transient exposure of testes to elevated temperatures (typically >40°C), surgical induction of cryptorchidism resulting in long-term exposure of testes to core body temperature (37°C), or housing of males at elevated temperatures (e.g., 35–36°C) for several hours [28–30]. We previously demonstrated that mild scrotal heat stress was associated with a temperature-dependent increase in DNA strand breaks, greater germ cell loss, and persistence of damaged DNA in sperm [6]. In the present study, we investigated the nature of the stress response in the mouse testis following transient scrotal heat. Our results demonstrate

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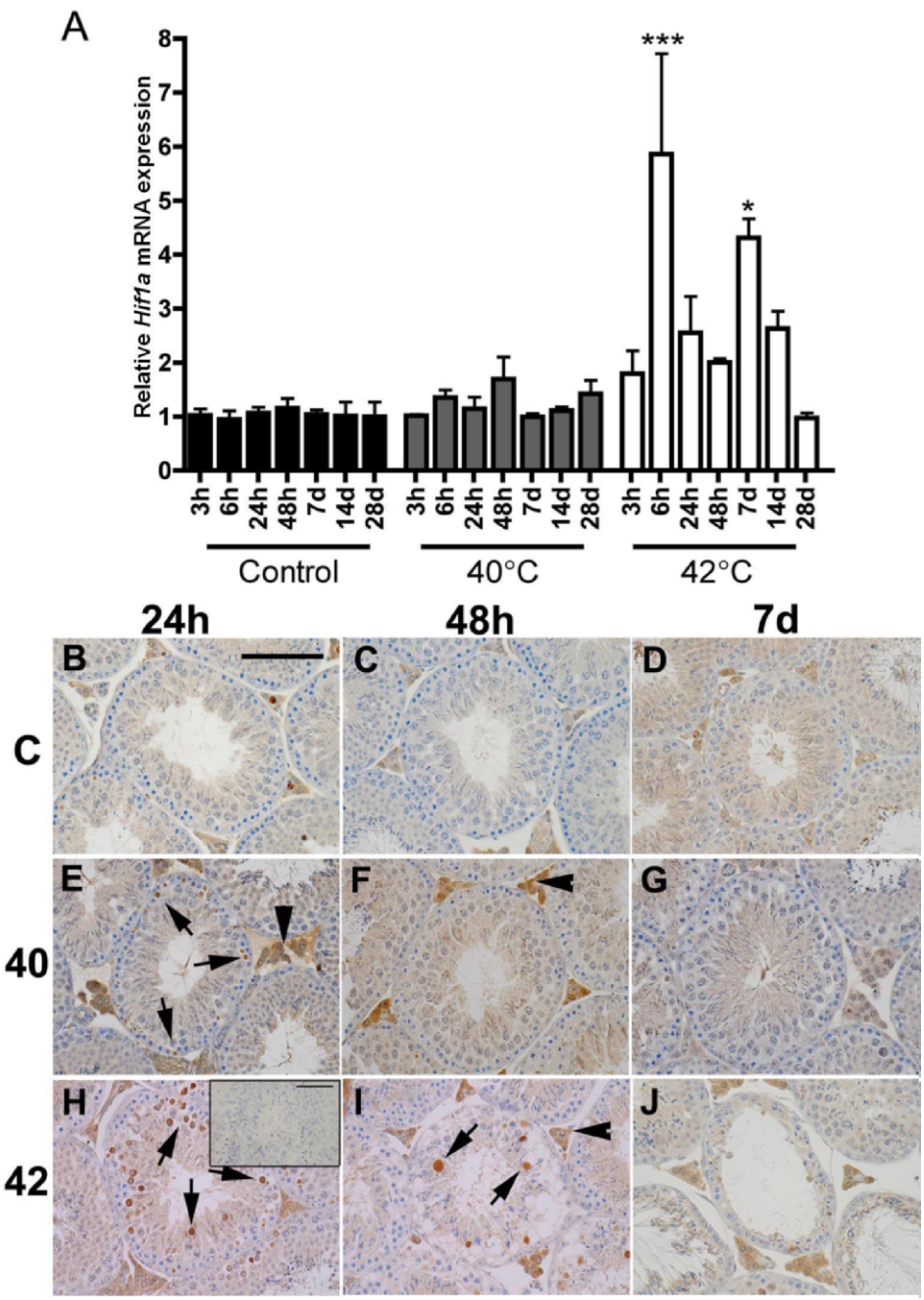

FIG. 1. *Hif1a* mRNA expression (**A**) and immunolocalization of protein (**B**–**J**) in testes from control and heat-stressed mice at 24 h (**B**, **E**, and **H** [inset shows negative control]), 48 h (**C**, **F**, and **I**), and 7 days (**D**, **G**, and **J**) after treatment. Arrowheads in panels **E**, **F**, and **I** identify immunopositive cells within the interstitium (presumptive Leydig cells), where the intensity of immunostaining appears increased compared with that in controls. Arrows show HIF1A-positive germ cells. Bar = 100 μm. $^{*}P < 0.05$, $^{***}P < 0.001$ (n = 4).

that transient mild hyperthermia causes hypoxia and oxidative stress and that this occurs in a temperature-dependent manner.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were maintained under standard conditions according to United Kingdom (UK) Home Office guidelines, with free access to food and water. All mice were purchased from Harlan Sprague-Dawley Inc. (Oxford, UK). Three hours to 28 days after heat shock, animals were killed by cervical dislocation.

Induction of Transient Heat Stress

Males aged 8–9 wk were subjected to a single heat stress of 40°C or 42°C for 30 min. Each animal was anesthetized, and the lower third of the body (hind legs, tail, and scrotum) was submerged in a water bath. Control animals were anesthetized and left at room temperature. After 30 min, each animal was administered an anesthetic reversal agent (Antisedan; Pharmacia and Upjohn, Corby, UK), dried, and returned to its cage.

Immunohistochemistry

One testis was immersion fixed in Bouin solution for 8 h. Sections (5 μm) were mounted on charged slides, and antigen retrieval was performed using 0.01 M citrate buffer (pH 6.0), with washes and blocking of endogenous peroxidase as described by Saunders et al. [31]. Nonspecific binding sites were blocked using normal goat serum (NGS) (Autogen Bioclear UK Ltd., Wiltshire, UK) diluted 1:4 in bovine serum albumin (BSA)/Tris-buffered saline (TBS) (5%, w/v) for 30 min. Sections were incubated overnight at 4°C with rabbit polyclonal antibodies specific for cleaved caspase 3 (Cell Signaling Technology, Beverly, MA) or HIF1A, both diluted 1:200 in NGS/TBS/BSA, and control sections were incubated with blocking serum alone. Bound antibodies were detected according to standard methods [31]. Counts of caspase 3-positive germ cells were made on a Provis AX70 microscope (Olympus Optical, London, UK). This was achieved

by counting the total number of positive cells in four testis sections per animal, obtained at least 50 μm apart. Images were captured from a microscope (BH2; Olympus Optical) under a 40× lens using a video camera (HV-C20; Hitachi Software Engineering, Yokohama, Japan).

RNA Extraction and Qualitative RT-PCR

Total RNA was extracted from adult testes from each control and heated group using the RNeasy Mini Kit with on-column DNase digestion (Qiagen Ltd., Crawley, West Sussex, UK). Random hexamer-primed cDNA was prepared using the Taqman RT kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed on the ABI Prism Sequence Detection System (Applied Biosystems). Expression of mouse *Hif1a* mRNA was determined using the Assay-on-Demand gene expression primers (catalog No. Mm00468878; Applied Biosystems), and heme oxygenase 1 (HO-1) was detected using the Roche Universal Probe Library (Roche Applied Sciences, Burgess Hill, UK) (forward primer: AGGCTAAGACCGCCTTCCT, reverse primer: TGTGTTCCTCTGTCAGCATCA, probe No. 17: AGGAGCTG). Expression levels of *Hif1a* and *Hmox1* were corrected using an endogenous control (18S rRNA, catalog No. 4308329; Applied Biosystems), and the fold differences in mRNA expression of samples were relative to the internal control sample, which was included in all runs. The results shown are the mean of at least four mice per group, performed on two separate occasions.

Western Blotting

Total protein was extracted from frozen testes using ristocetin-induced platelet agglutination lysis buffer, and protein concentration was determined using a protein assay kit (BioRad; Hemel Hempstead, UK) according to the manufacturer's instructions. Samples (20 μg/lane) were separated on 4%–12% polyacrylamide (w/v) gradient gels (Invitrogen, Paisley, UK) at 200 V for 1 h and then transferred onto polyvinylidene fluoride membranes. Proteins were detected using antibodies specific for HMOX1 (1:200), GPX1 (1:250), GST alpha (GSTA) (1:3000), or ICAD (1:500) (Abcam, Cambridge, UK), all diluted with Odyssey buffer (LiCor, Lincoln, NE). Bound antibodies were detected using an analysis system (LiCor) as described in detail by Anderson et al. and were compared with the amount of β-actin (ACTB) (1:1000; Abcam) to correct for loading.

Statistical Analysis

Results expressed as means ± SEMs were analyzed using one-way ANOVA, followed by Bonferroni post hoc test. GraphPad Prism version 4 (Graph Pad Software Inc., San Diego, CA) was used for analysis.

RESULTS

Heat Stress-Induced Hypoxia in the Testis

Heat stress at 40°C had no effect on expression of mRNA encoded by the hypoxia-related gene *Hif1a* compared with that in controls (Fig. 1A). However, scrotal heating at 42°C induced a significant (6 fold) increase in *Hif1a* mRNA expression at 6 h ($P < 0.001$), which decreased to approximately twice that of controls and subsequently increased again to 4-fold that of controls at 7 days ($P < 0.05$).

Under normoxic conditions, HIF1A protein is typically localized in the cytoplasm; however, under hypoxic conditions proteosome-dependent degradation is reduced, and HIF1A translocates to the nucleus, where it is able to activate downstream genes, including vascular endothelial growth factor and lactate dehydrogenase A [33, 34]. In control testes, cytoplasmic staining was detected in the interstitium and seminiferous epithelium, and no nuclear immunostaining was observed (Fig. 1). Following heat stress at 40°C or 42°C, the intensity of immunostaining in the interstitial compartment seemed to be increased compared with that in controls (e.g., Fig. 1, E, F, and I [arrowheads]). Nuclear staining was detected in a few germ cells located at the periphery of the tubule in the 40°C treatment group at 24 h (Fig. 1D, arrows). In the 42°C treatment group, immunopositive staining for HIF1A was localized to germ cell nuclei within the seminiferous tubules at

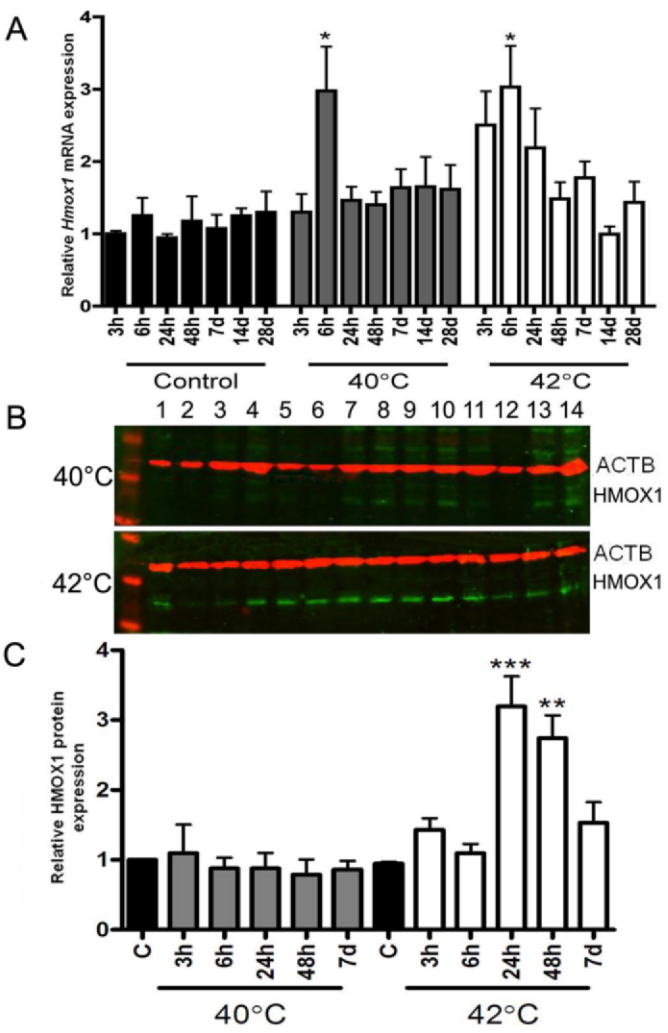

FIG. 2. *Hmox1* mRNA (**A**) and protein expression (**B** and **C**) in testes from control and heated (40°C and 42°C) mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **B**) Representative Western blot showing controls (n = 4) and heated testis samples (n = 2). Lanes 1–4: controls; lanes 5 and 6: 3 h; lanes 7 and 8: 6 h; lanes 9 and 10, 24 h; lanes 11 and 12: 48 h; and lanes 13 and 14: 7 d after heat treatment. Panel **C** is generated from data from heated testis samples (n = 4).

24 h (Fig. 1H), and although some immunopositive nuclei were also detected 48 h after heat stress, the numbers were reduced compared with those at the earlier time point, consistent with substantial germ cell loss resulting in "gaps" within the epithelium at this time point (Fig. 1I).

Heat Stress-Induced Oxidative Stress

The effect of heat stress on expression of *Hmox1* mRNA, which is reported to be expressed in Sertoli cells and Leydig cells after heat shock [35], was measured using quantitative RT-PCR. Transient heat stress at 40°C and 42°C was sufficient to cause upregulation of *Hmox1* expression (Fig. 2A). A significant increase was observed at 6 h in the 40°C treatment group ($P < 0.05$). In the 42°C treatment group, expression had already increased by 3 h and remained elevated at 24 h, but by 48 h the mRNA levels in both treatment groups had decreased to control levels. Heat stress at 40°C did not induce any change in total HMOX1 protein expression at any of the time points measured (Fig. 2, B and C). However, in the 42°C treatment group a significant increase in total HMOX1 protein expression

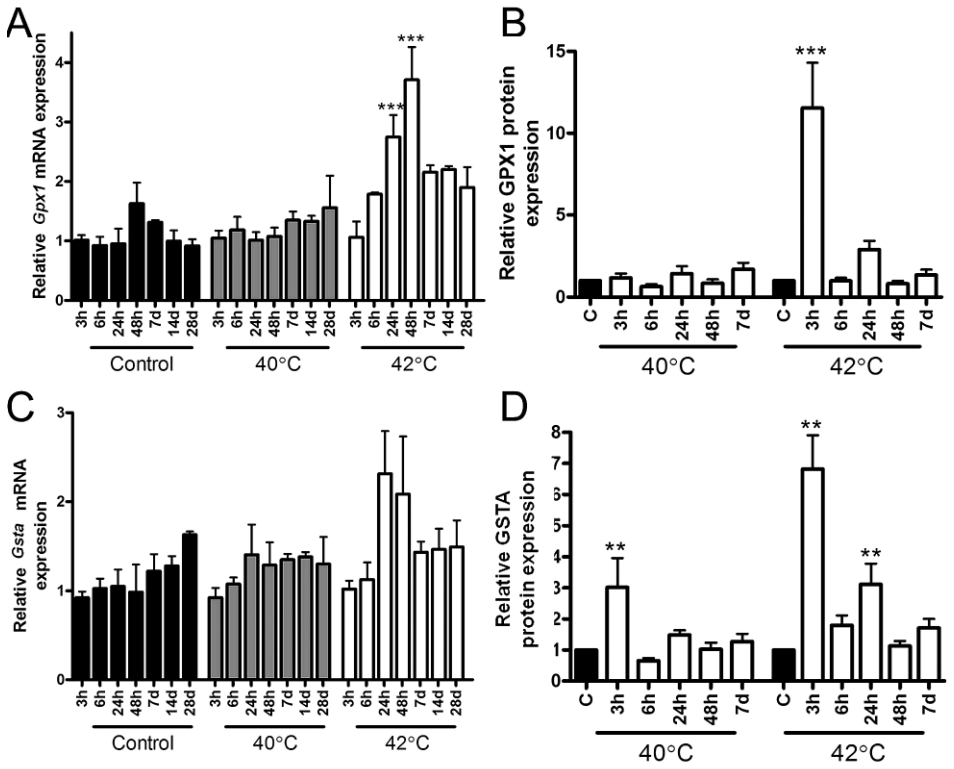

FIG. 3. Expression of *Gpx1*/GPX1 (**A** and **B**) and *Gst*/GST (**C** and **D**) antioxidants (*Gsta*/GSTA) in control and heated (40°C and 42°C) testes. $^{**}P < 0.01$, $^{***}P < 0.001$ (n = 4).

was detected in testes at 24 h ($P < 0.001$) and at 48 h ($P < 0.01$) compared with than in controls (Fig. 2, A and C).

Heat stress also led to increased expression of two other antioxidant enzymes, GPX1 and GST (Fig. 3). *Gpx1* mRNA was increased significantly only in the 42°C treatment group at 24 h and at 48 h (Fig. 3A). However, protein levels were significantly increased compared with those in controls at the earlier time point of 3 h ($P < 0.001$) (Fig. 3B). *Gsta* mRNA levels were also slightly increased at 24 h and 48 h, although this was not significant (Fig. 3C). However, heat stress at 40°C and 42°C was sufficient to cause an increase in protein levels at 3 h, and 42°C caused a second increase in GST protein level at 24 h ($P < 0.01$ for all) (Fig. 3D).

Caspase 3-Mediated Apoptosis Is Activated Following Scrotal Heat Stress

Testicular sections were stained for cleaved caspase 3 to determine whether there was any change in expression of this effector caspase (Fig. 4A). Although many of the caspase 3-positive cells were located close to the basement membrane and were likely to be spermatogonia, some immunopositive spermatocytes were also present in testes recovered from the 40°C and 42°C treatment groups. Previous findings suggested that spermatogonial stem cells are not susceptible to hyperthermia [36]. The 40°C treatment group exhibited an 11-fold increase in caspase-positive cells at 24 h, but this had decreased to 3-fold by 48 h (Fig. 4A). In the 42°C treatment group, there was a 9-fold increase at 6 h, and at 24 h there was a significant 27-fold increase in the number of positive cells ($P < 0.01$). This was still more than 10 times that of controls at 48 h. Protein expression of ICAD was also determined (Fig. 4B). Heat stress at 40°C did not induce any changes in expression, but a significant decrease in ICAD protein expression was seen at 6 h and 24 h ($P < 0.05$ for both) following 42°C heat stress.

DISCUSSION

Heat stress can disrupt spermatogenesis, causing germ cell death and subfertility [1, 4, 6, 37], and elevated testicular temperature has been suggested as a possible contraceptive treatment for men [38, 39], although there may be several undesirable adverse effects associated with this treatment. In a previous study [6], we reported that hyperthermia resulted in a number of detrimental effects on the testis, including DNA damage in germ cells and mature sperm and a lengthy recovery period of the testis from mild transient heat shock. The objective of the present study was to elucidate the response of the testis to this stress, which ultimately leads to the previously observed DNA damage and germ cell loss. This was achieved by looking at markers of hypoxia, oxidative stress, and apoptosis.

A 1988 study [9] assessing testicular hyperthermia in rats demonstrated a reduction in blood flow into the testis when heated to 42°C and 43°C. Because the testis relies on blood flow to provide oxygen, we examined whether a hyperthermic stress model resulted in exposure of testes to hypoxic conditions. HIF is a heterodimeric DNA-binding complex expressed in all mammalian cells; it consists of an oxygen-regulated α subunit and an oxygen-independent β subunit. Under normoxic conditions, HIF1A subunits are rapidly degraded via the ubiquitin-proteasome pathway; however, when oxygen levels decrease, the protein is stabilized [40] and translocates from the cytoplasm to the nucleus, where it dimerizes with ARNT (also known as Hif1β) [41, 42].

In this study, expression of *Hif1a* mRNA and protein in testes heated to 40°C and 42°C was compared with that in controls housed under identical conditions. In the controls, HIF1A protein was detected in the cytoplasm of interstitial cells and in elongate spermatids, consistent with a previous study [43] demonstrating that in situ hybridization localized various isoforms of *Hif1a* mRNA to spermatids and other cell

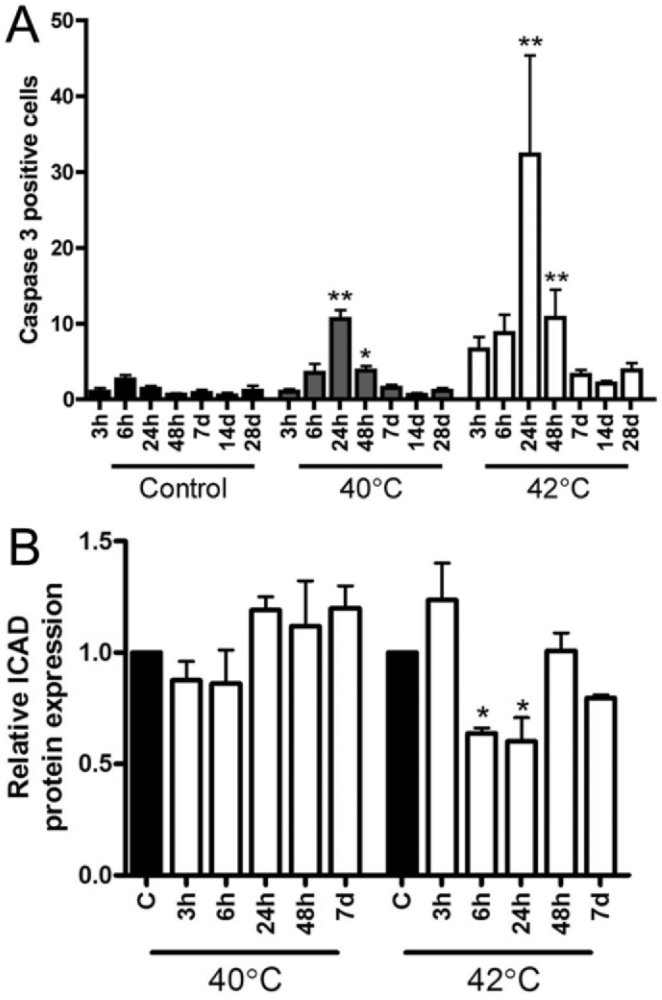

FIG. 4. Expression of apoptosis-related proteins (cleaved caspase 3 [**A**] and ICAD [**B**]) in control and heated (40°C and 42°C) testes. $^{*}P < 0.05$, $^{**}P < 0.01$ (n = 4).

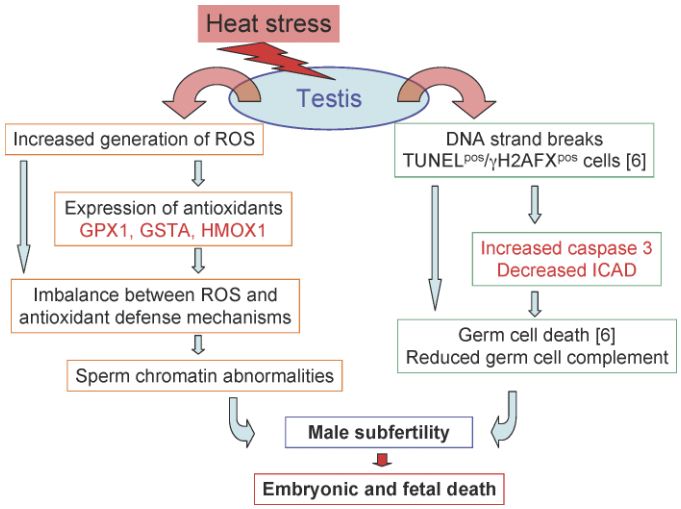

FIG. 5. Summary diagram of the possible mechanisms contributing to germ cell death following scrotal heat stress.

types within the seminiferous epithelium. Transient heat stress resulted in increased expression of *Hif1a* mRNA, apparent increase in the intensity of immunostaining in the interstitial compartment, and detection of immunopositive staining in the nuclei of germ cells. In samples from testes recovered from testes previously heated to 42°C, the nuclei of spermatocytes seemed to be immunopositive 24 h after heating. In our experience, apoptotic germ cells can often exhibit "false-positive" immunostaining reactions, but careful examination of the sections herein revealed spermatocytes with the morphological appearance of apoptotic cells that contained no HIF1A in their nuclei and would be consistent with stabilization and nuclear translocation within selected germ cells. Although stabilization of HIF1A protein under hypoxic conditions has been widely reported, less attention has been paid to changes in transcription of the *Hif1a* gene; therefore, it is notable that we detected a significant transient increase in *Hif1a* mRNA 6 h after heating at 42°C. Total concentrations of mRNA were also elevated 7 d after stress, and we assume that this was due to continued elevation in expression within somatic cells at a time when germ cell loss had occurred; therefore, the somatic mRNAs were not diluted by those from germ cells (Fig. 1J).

To our knowledge, this is the first study to show heat stress-induced hypoxia in the testis and to postulate that the reason the testicular tissue becomes hypoxic is that heat-induced increases in cell metabolism are not being matched by a sufficient increase in blood flow to maintain an adequate level of oxygenation. Although an initial study in rats by Galil and Setchell [9] demonstrated reduced blood flow into the testis in response to heat stress, further findings in rams showed an increase in blood flow as a result of local heating [44]. Further investigations are required to confirm whether the present experimental protocol had an effect on blood flow into mice testes.

Oxidative stress, which has been shown to cause damage to membranes, proteins, RNA, and DNA [45], was also investigated in the present study. The testis expresses several antioxidants, including SOD, GSR, GST, GPX, and HMOX1, that have the capacity to act as ROS scavengers and serve to protect the tissue and its resident germ cells from damage caused by oxidative stress [21, 46]. If the balance between ROS generation and scavenging is disrupted (i.e., the amount of ROS exceeds that of the ROS scavenging capacity), testicular function can be disturbed. For example, treatment with pro-oxidants such as organic hydroperoxide results in abnormal sperm production and reduced litter size, consistent with a significant effect on DNA integrity [47]. In humans, oxidative stress from smoking has been shown to cause increase oxidation of sperm DNA and poor antioxidant levels in semen, suggesting an association between oxidative damage and male reproductive dysfunction [48, 49] that can be transmitted to offspring [50]. Cryptorchidism, a condition that leads to the testis being exposed to abdominal temperatures, causes an increase in ROS production [51], and cells isolated from testes of 40-day-old rats produced increased levels of peroxide when heated to 43°C for 1 h [52].

In the present study, increased expression of testicular antioxidants (HMOX1, GPX1, and GSTA) in testes subjected to hyperthermia is consistent with a robust oxidative stress response. It is possible that increased expression of HIF1A may contribute to the observed increase in HMOX1, as the *Hmox1* gene is reported to contain elements that bind specifically to *Hif1* to mediate hypoxia-induced gene activation [53]. Although an increase in *Hmox1* mRNA was detected in testes retrieved 6 h after heating at 40°C, this was independent of any obvious change in expression of *Hif1a* in these samples, suggesting that the upregulation of *Hmox1* is more likely to be

in response to the production of ROS or other free radicals in the tissue. Changes in expression of the antioxidant enzymes GPX1 and GSTA were also detected. In both cases, an increase in total protein was detected only 3 h after heating at 42°C, whereas increases in mRNA (significant in the case of *Gpx1*) were detected at 24 h and 48 h, suggesting that heating resulted in protein stabilization or stimulated translation of preexisting transcripts. The former may be more likely, as several proteins that are involved in the response to oxidative stress seem to be regulated by stabilization (e.g., inducible nitric oxide synthase or NOS2 [54]), and these findings support our suggestion that hyperthermia is causing oxidative stress within the testis.

It is known that hypoxia leads to cell cycle arrest and apoptosis [12, 13], and oxidative stress has recently been shown to cause depletion of germ cells [55]. We previously demonstrated that thermal heat stress results in increased numbers of DNA strand breaks in spermatocytes and in increased numbers of germ cells with TUNEL-detected DNA fragmentation [6]. In the present study, we examined whether this process may be mediated via caspase 3, one of a group of effector caspases. An increase in the number of germ cells that were immunopositive for cleaved caspase 3 was detected 24 h after heat shock at 40°C or 42°C, demonstrating that expression of this protein is sensitive to slight fluctuations in temperature. In addition, we detected a significant decrease in protein expression of ICAD, a specific inhibitor of CAD, which is a DNase believed to degrade DNA during apoptosis [26]. In apoptosis, caspase 3 cleaves ICAD, releasing CAD to take part in DNA degradation [27]; therefore, we suggest that reduced expression of ICAD may have further amplified the apoptotic signal.

Our studies used a model of acute transient thermal stress, and although this might mimic the situation of a man taking a hot bath, it begs the question as to what effect living in a hot climate might have on testicular function. Zhu and Setchell [28] attempted to address this question by using an alternative mouse model in which males were housed for 24 h to an ambient temperature of 36 ± 0.3°C and a relative humidity of 66% ± 5.6%. In these mice, heat stress 35 days before mating decreased the proportion of normal embryos and blastocysts. Cryptorchidism (natural or induced) in which testes are kept at 37°C for extended periods results in germ cell apoptosis, seminiferous tubule vacuoles, and reduced testis weight [56, 57]. However, the testis is clearly capable of an adaptive stress response, as illustrated by the increased expression levels of HMOX1 in the testis of patients with varicocele [58], and even slight increases in HIF1A expression could have a major effect on the ability of testicular cells to adapt to reduced oxygen levels [40].

In conclusion, using a model of transient thermal stress, we and others have detected increased expression of caspase 3 and increased incidence of unrepaired double-strand DNA breaks [6] and greater germ cell loss, possibly in part as a result of reduced expression of DNA repair genes [59]. Although we have shown that the testicular response to thermal stress includes induction of HIF1A (consistent with hypoxia) and increased expression of antioxidants (consistent with oxidative stress), germ cell death still occurs. Therefore, one explanation is that these changes are insufficient to compensate for increased levels of ROS generated within the testis and epididymis and that all of these factors contribute to reduced fertility (Fig. 5). It is notable that oxidative stress has been suggested as a common cause of male infertility and that increased use of antioxidant therapies has been proposed [60]. Our studies provide further evidence that modest acute thermal stress may contribute to male subfertility.

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